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In Vitro Combination of Anidulafungin and Voriconazole against Intrinsically Azole-Susceptible and -Resistant *Aspergillus* spp.

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***In vitro* interaction of anidulafungin with voriconazole was tested by a microdilution broth checkerboard technique and an agar diffusion method against 30 *Aspergillus* clinical isolates belonging to five different species. By using a complete inhibition endpoint, indifferent interactions were observed for 97% of the isolates by the checkerboard technique (FIC index from 0.5 to 2) and for 100% of the isolates by the agar diffusion method (variation of −2 to +1 $\log_2$ dilutions).**

Voriconazole is the first-line therapy for invasive aspergillosis (19, 37). Nevertheless, mortality remains high due to different factors (4, 7, 21, 24, 27). Although azoles are very active *in vitro* against *Aspergillus fumigatus* (18), several studies have reported *de novo* or acquired azole resistance (9, 11, 15). While in selected populations of patients the frequency of these azole-resistant isolates may be low (2, 13), an emergence of azole resistance has been reported in Europe in clinical and environmental *A. fumigatus* isolates (8, 31, 34). Moreover, other pathogenic *Aspergillus* species are naturally azole resistant (6, 33). For these emerging species (26, 35), first-line voriconazole therapy may not be recommended (37), and therefore, combination therapy may be of interest.

Since a clinical trial evaluating the efficacy of voriconazole plus anidulafungin versus voriconazole as first-line treatment in invasive aspergillosis has recently been completed, we evaluated the *in vitro* interaction of voriconazole with anidulafungin against different *Aspergillus* species.

Thirty *Aspergillus* clinical isolates belonging to intrinsically azole-susceptible and -resistant species (11 *A. fumigatus*, 5 *A. flavus*, 5 *A. terreus*, 5 *A. calidoustus*, 3 *A. nidulans*, and 1 *A. sydowii* isolates) were tested. Species identification was performed by sequencing the beta-tubulin and/or calmodulin gene (1) as recommended (5). Drug combinations were tested by two different techniques: a broth microdilution checkerboard procedure based on the Clinical and Laboratory Standards Institute (CLSI) M38–A2 document (10) and an agar diffusion test (Etest) (36).

For checkerboard studies, the final concentrations of voriconazole (Pfizer Inc., New York, NY) and anidulafungin (Pfizer) were 0.03 to 2 μg/ml and 0.0001 to 0.06 μg/ml, respectively. Spore suspensions were counted in a hemocytometer and adjusted to the required concentration. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included as quality controls. MICs were determined visually after 48 h of incubation at 35°C in two independent experiments. At first, the MICs were determined with a complete inhibition endpoint. Alternatively, a partial inhibition endpoint (50% inhibition for voriconazole and minimum effective concentrations [MECs], determined as previously described [3] for anidulafungin alone or for both drugs in combination) was also used. Fractional inhibitory concentration (FIC) indices (16) were calculated, and drug interactions were defined as synergistic, additive (i.e., no interaction/indifferent), or antagonistic when the FIC index was ≤0.5, >0.5 and ≤4, or >4, respectively (25).

Antifungal susceptibility was also evaluated by the Etest (AB Biodisk, Solna, Sweden). RPMI agar plates were inoculated with a spore suspension adjusted to 10^6 conidia/ml. For combination studies, anidulafungin Etest strips were placed on RPMI agar, the strips were discarded after 1 h, and voriconazole strips were placed at the same position. After incubation at 35°C for 48 h, MICs were determined visually with either a complete or partial inhibition endpoint (17). Experiments were run in duplicate. Synergy or antagonism was defined, respectively, as a decrease or an increase of ≥3 dilutions of the resultant MIC (20).

The activity of voriconazole and anidulafungin either alone or in combination was first determined by checkerboard microdilution (Table 1). Voriconazole MICs ranged from 0.06 to 4 μg/ml, with differences between species. Voriconazole MICs against *A. fumigatus*, *A. flavus*, and *A. terreus* ranged from 0.25 to 1; those of *A. calidoustus* were higher, ranging from 2 to 4 μg/ml. All isolates from all species exhibited low anidulafungin MECs (range, 0.001 to 0.06 μg/ml). Overall, and whatever the triazole susceptibility, the combination of voriconazole and anidulafungin showed no interaction (FIC indices between 0.50 and 2) for 97% of the isolates by using a complete inhibition (MIC, 0) endpoint. A synergistic interaction was observed for only one *A. sydowii* isolate (FIC, 0.5). When a less stringent endpoint (MEC) was used, a synergistic interaction was observed for one *A. calidoustus* isolate (FIC, 0.28) and an antagonistic interaction was observed for two *A. flavus* isolates (FIC,

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TABLE 1 Drug interaction of voriconazole in combination with anidulafungin against *Aspergillus* spp. by the checkerboard microdilution broth technique[a]

Species (no. of isolates)	MIC/MEC range (GM) (μg/ml) of the drugs alone[b]		FIC index range (GM) for the combination VRZ/ANI[c]	
	VRZ	ANI	MIC-0	MEC
A. fumigatus (11)	0.25 (0.25)	0.001–0.03 (0.012)	0.56–1 (0.73)	1–1.5 (1.24)
A. flavus (5)	0.5–1 (0.87)	0.008–0.06 (0.024)	0.62–1 (0.86)	0.75–4.5 (2.07)
A. terreus (5)	0.5 (0.5)	0.008–0.015 (0.01)	0.75–1 (0.94)	1.12–2.12 (1.28)
A. calidoustus (5)	2–4 (3.48)	0.008–0.06 (0.024)	1–2 (1.74)	0.28–2 (1.19)
A. nidulans (3)	0.06–0.125	0.002–0.008	1	1–9
A. sydowii (1)	0.25	0.015	0.5	1.5
All species (30)	0.06–4 (0.48)	0.001–0.06 (0.014)	0.5–2 (0.92)	0.28–9 (1.47)

[a] The drug interaction of voriconazole in combination with anidulafungin against 30 isolates of *Aspergillus* spp. was determined by the checkerboard microdilution broth technique using two different endpoints. GM, geometric mean; VRZ, voriconazole; ANI, anidulafungin.
[b] MIC and MEC were determined visually as the concentration that gave 100% of inhibition (MIC-0) for VRZ and abnormal hyphal growth (MEC) for ANI.
[c] Corresponding to the lowest FIC index.

4.5) and one *A. nidulans* isolate (FIC, 9), whereas no interaction (FIC indices between 0.75 and 2.5) was observed for 87% of the isolates. MICs of the replicates were within ±1 $\log_2$ dilution in 88% of the cases. The results of agar diffusion tests are shown in Table 2. Voriconazole MICs ranged from 0.03 to 8 μg/ml, with higher MICs against *A. calidoustus* (range, 2 to 8 μg/ml) than against *A. fumigatus*, *A. flavus*, and *A. terreus* (range, 0.125 to 0.5 μg/ml). All isolates exhibited low anidulafungin MICs (range, 0.002 to 0.008 μg/ml). In combination, by using a complete inhibition endpoint, voriconazole and anidulafungin showed no interaction: for all isolates, the voriconazole MICs in combination were within ±2 $\log_2$ dilutions of the voriconazole MICs tested alone. With a partial inhibition endpoint, no interaction was observed for 93% of the isolates and an increase of 3 $\log_2$ dilutions was noted for two *A. calidoustus* isolates. Typical patterns observed by Etest are shown in Fig. 1. MICs were within ±1 $\log_2$ dilution for all the replicates.

In the present study, the combination of voriconazole and anidulafungin rarely showed an interaction. Because *in vitro* antifungal interaction against filamentous fungi remains difficult to test (23), we used two unrelated techniques. Although the Etest assesses interactions at a certain concentration ratio of drugs in combination whereas the checkerboard microdilution assay evaluates interactions at different concentration ratios, the results obtained by the two techniques were globally similar. The Etest was used previously for testing antifungal combinations (12, 14, 20), but this is the first study, to our knowledge, that used the Etest to evaluate the interaction between anidulafungin and voriconazole. By including intrinsically azole-susceptible and -resistant *Aspergillus* species, we demonstrated that the lack of interaction between the two drugs is not dependent on the azole susceptibility of the isolates.

Previous studies evaluating the interaction between anidulafungin and voriconazole against *Aspergillus* spp. showed conflicting results (28–30, 32). In one *in vitro* study, almost no interaction was found against different species of *Aspergillus* (28). In another study, synergistic interactions were found (30). The differences between studies may be related to different methodological approaches. In particular, the limitations of the present study may be related to the visually determined MIC endpoints, which may be subjective, particularly for combinations with echinocandins, and the wide range of FIC index cutoffs used to detect synergy. Recently, a randomized trial evaluating the efficacy of anidulafungin and voriconazole in combination for primary therapy of invasive aspergillosis was completed and showed that the combination was not associated with a lower risk of early mortality compared to voriconazole alone (22).

In conclusion, our results showed that combination of anidu-

TABLE 2 Drug interaction of voriconazole in combination with anidulafungin against *Aspergillus* spp. by Etest[a]

Species (no. of isolates)	MIC range (GM) (μg/ml) of the drugs alone[b]		MIC range (GM) (μg/ml) of the combination VRZ/ANI		Variation range (median) of MIC ($\log_2$ dilutions)[c]	
	VRZ	ANI	Complete inhibition	Partial inhibition	Complete inhibition	Partial inhibition
A. fumigatus (11)	0.125 (0.125)	0.002–0.008 (0.003)	0.06–0.125 (0.10)	0.004–0.008 (0.006)	−1–0 (0)	0–+2 (+1)
A. flavus (5)	0.25–0.5 (0.33)	0.002–0.004 (0.002)	0.125–0.25 (0.19)	0.004 (0.004)	−1–0 (−1)	0–+1 (+1)
A. terreus (5)	0.125–0.5 (0.25)	0.002–0.004 (0.003)	0.125–0.25 (0.14)	0.002–0.004 (0.003)	−1–0 (−1)	0–+1 (0)
A. calidoustus (5)	2–8 (2.64)	0.002–0.004 (0.003)	0.5–4 (1)	0.002–0.015 (0.010)	−2–−1 (−1)	0–+3 (+2)
A. nidulans (3)	0.03–0.06	0.002–0.004	0.03–0.06	0.002–0.004	−1–+1	0–+1
A. sydowii (1)	0.125	0.002	0.125	0.002	0	0
All species (30)	0.03–8 (0.25)	0.002–0.008 (0.003)	0.003–4 (0.16)	0.002–0.015 (0.005)	−2–+1 (−1)	0–+3 (+1)

[a] The drug interaction of voriconazole in combination with anidulafungin against *Aspergillus* spp. was determined by Etest using two different endpoints. GM, geometric mean; VRZ, voriconazole; ANI, anidulafungin.
[b] MICs were determined visually as the concentration that gave 100% of inhibition for VRZ and partial inhibition for ANI.
[c] Number of $\log_2$ dilution differences between MIC of the drug alone and in combination.

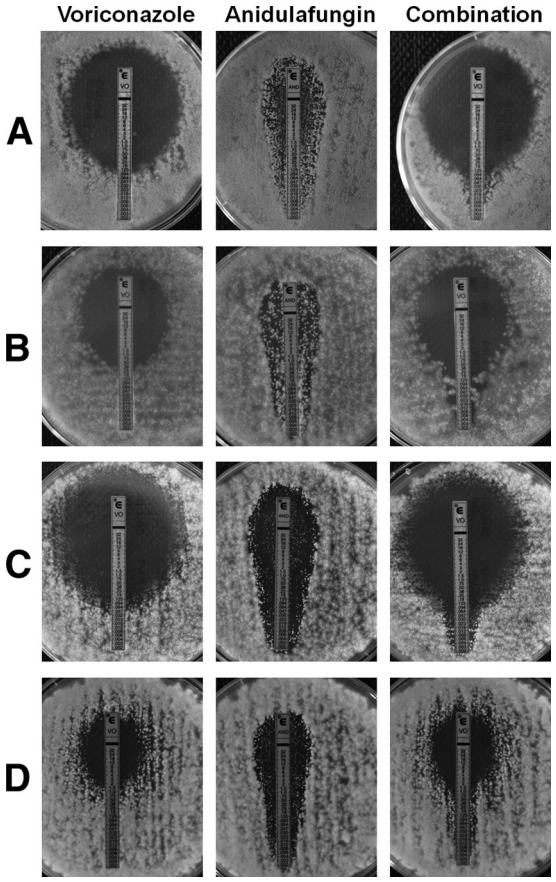

FIG 1 Agar diffusion test of the combination of voriconazole with anidulafungin against *A. fumigatus* FUM02 (row A), *A. flavus* FLA05 (row B), *A. terreus* TER01 (row C), and *A. calidoustus* UST01 (row D). For combination tests, an anidulafungin Etest strip was placed on the agar surface, left for 1 h, and removed, and a voriconazole strip was then applied.

lafungin with voriconazole is not synergistic *in vitro* against various triazole-susceptible or -resistant *Aspergillus* species.

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